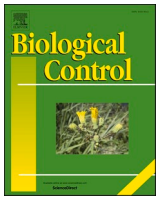
Biological Control 212 (2026) 105949

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



Growth-promoting and biocontrol features of *Pantoea ananatis* BRT175 in tomato

Simon Duchateau [a,1], Célia Borrego [a,1], Sonia Verdelet [a], Stéphan Dorey [a], Aziz Aziz [a], Sandrine Dhondt-Cordelier [a], Charles Gauthier [b], Éric Déziel [b], Sylvain Cordelier [a,2], Jérôme Crouzet [a,*,2]

[a] University of Reims Champagne-Ardenne, INRAE, RIBP USC 1488, 51100 Reims, France
[b] INRS-Centre Armand-Frappier Santé Biotechnologie, Laval, Québec, Canada

HIGHLIGHTS

- *Pantoea ananatis* BRT175 presents PGPR and biocontrol traits *in vitro*.
- It promotes tomato growth in both growth chamber and greenhouse conditions.
- It protects tomato against *Botrytis cinerea* at both local and systemic scales.
- Ananatosides contribute to the antifungal activity of this strain.

ARTICLE INFO

Keywords:
Pantoea ananatis
Tomato
PGPR
ISR
Biocontrol
Botrytis cinerea
Ananatosides

ABSTRACT

Beneficial bacteria belonging to *Pantoea* spp. and their interactions with plants have recently attracted growing interest for their beneficial effects, especially in promoting plant growth and health. In this study, we evaluated the growth-promoting and induced resistance activities of *Pantoea ananatis* strain BRT175 in tomato. *In vitro* assays revealed that this strain exhibits different beneficial traits, including phosphate solubilization, siderophore production, and IAA synthesis. These traits were further supported *in silico* by the presence of corresponding genes annotated in *P. ananatis* BRT175 genome. The capacity of this strain to significantly promote tomato growth was demonstrated under both growth chamber and greenhouse conditions. This bacterium also showed significant biocontrol activity through its antifungal effect against *Botrytis cinerea*. Interestingly, *P. ananatis* BRT175-derived ananatosides, a group of amphiphilic glycolipids, also showed an antifungal effect against *B. cinerea*. These glycolipids could therefore act as bacterial determinants contributing to protection against the pathogen. In addition, both root and leaf treatments with the bacterium resulted in a significant reduction of necrotic symptoms, suggesting that *P. ananatis* BRT175 potentially triggers systemic resistance of tomato. At the leaf level, *P. ananatis* BRT175 may displays a multifaceted protective effect by combining antifungal properties, competition for nutrients, and stimulation of tomato systemic resistance.

1. Introduction

Plants are in a wide array of continuous interactions with bacteria in their natural environment (Trivedi et al. 2020; Compant et al. 2019). They are fully colonized by bacteria, which can be associated with the root system or the aerial organs, either within (endophytes) or outside (epiphytes) plant tissues (Kandel et al. 2017; Bacon and White, 2016; Compant et al. 2010). Among these microbial colonizers, some genera exhibit beneficial traits for plants. For instance, bacteria from the *Pseudomonas*, *Bacillus*, *Burkholderia*, *Rhizobium*, and *Azospirillum* genus are well-known for their beneficial effects, either on plant health and/or plant growth (Dutilloy et al. 2024; Mehmood et al. 2023; Nguyen et al.

* Corresponding author.
E-mail address: jerome.crouzet@univ-reims.fr (J. Crouzet).
[1] These authors contributed equally to this work.
[2] Co-last authors.

https://doi.org/10.1016/j.biocontrol.2025.105949
Received 18 July 2025; Received in revised form 21 October 2025; Accepted 14 December 2025
Available online 15 December 2025

2022; Saxena et al. 2020; Fukami et al. 2018; Gruau et al. 2015; Suárez-Moreno et al. 2012). These plant growth-promoting (rhizo)bacteria (PGPR or PGPB) enhance plant performance through several mechanisms. Regarding plant growth, PGPR enhance plant performances of their host through biosynthesis of plant hormones like auxins (Pantoja-Guerra et al. 2023; Grover et al. 2021; Hayat et al. 2010). Some PGPR also have the ability to solubilize phosphorus from inorganic sources present in the soil, especially in mineral form or bound to cations (Bargaz et al. 2021; Rawat et al. 2021). Through soil acidification or phosphatase synthesis, beneficial bacteria mobilize inorganic phosphorus which can then be available to plants in an assimilable form (Rawat et al. 2021). Diazotrophic bacteria are another example of beneficial microbes that enhance plant nutrition by fixing atmospheric nitrogen and converting it into nitrates (Zeng et al. 2022). Regarding plant health, some beneficial bacteria can exhibit antagonistic potential against various plant pathogens, either through synthesis of antimicrobial compounds (Dimkić et al. 2022; Raaijmakers and Mazzola, 2012) or through competition for nutrients (Köhl et al. 2019; Berendsen et al. 2012). Some rhizobacteria are also able to stimulate plant immunity to combat pathogen spread. This state of enhanced defense is called Induced Systemic Resistance (ISR) (Yu et al. 2022, Pieterse et al. 2014; Van Wees et al. 2008), and is the outcome of a combined process involving bacteria perception at the root level (De Vleesschauwer et al., 2009; Van Loon and Bakker, 2006), and the systemic spread of resistance across all organs through phytohormone signaling pathways (Vlot et al. 2021).

The beneficial potential of bacteria belonging to the *Pantoea* genus has been explored during recent years (Duchateau et al. 2024; Walterson and Stavrinides, 2015). In particular, several strains of *P. ananatis* and *P. agglomerans* have shown promising potential as biocontrol agents or plant growth promoters (Valbuena-Rodríguez et al. 2024; Lu et al. 2021; Kim et al. 2012). The present research work focuses on the strain *P. ananatis* BRT175, an epiphytic isolate from strawberry (Smith et al. 2013). To date, this strain has been investigated for its capacity to produce biocontrol-related molecules such as ananatosides, a group of rhamnolipid analogues exhibiting eliciting properties (Cloutier et al. 2021; Gauthier et al. 2019), and PNP-1, an antibiotic effective against *Erwinia amylovora* (Okrent et al. 2018). In this study, we assess the ability of this strain to promote the growth of tomato, a food crop of economic interest. As fungal pathogens opposed major threats to tomato production, we also assay the capacity of the bacterium to protect this plant against *Botrytis cinerea*, the causal agent of grey mold. The mechanisms and mode of action involved in the beneficial features of *P. ananatis* BRT175 are also addressed in this study by assaying different properties of this strain, like siderophore production or auxin synthesis for instance.

2. Material and methods

2.1. Microorganisms and growth conditions

Precultures of *Pantoea ananatis* strain BRT175 were prepared in 10 mL Lysogeny Broth (LB) supplemented with 50 μg.mL^{-1} rifampicin and inoculated with 250 μL of glycerol stock. Precultures were incubated overnight at 28 °C under continuous shaking (180 rpm). Cultures were prepared in 150 mL of LB medium supplemented with rifampicin and inoculated with 5 mL of preculture. After overnight incubation (28 °C, 180 rpm), bacteria were collected by centrifugation (3500 *g* for 15 min at 8 °C). Pellets were resuspended in 5 mL of sterile 10 mM $MgSO_4$ solution and the bacterial concentration was adjusted to 10^9 CFU.mL^{-1}.

Escherichia coli TOP 10 (Invitrogen) was used as a bacterial negative control for *in vitro* assays. Cultures were prepared as described above, in 150 mL LB supplemented with ampicillin 50 μg.mL^{-1}.

Botrytis cinerea strain 630 (*Bc*630) (INRAE, Versailles, France) was initially grown in 25 mL of the following medium ($MgSO_4$ 750 mg.L^{-1}, KH_2PO_4 175 mg.L^{-1}, glucose 400 mg.L^{-1}, peptone 400 mg.L^{-1}, citric acid 192 mg.L^{-1}, Tween 20 500 μL.L^{-1}, pH 3.1) by adding 250 μL of glycerol stock containing spores. After 7 days of incubation at 20 °C and 140 rpm with a 16/8h photoperiod (80 μmol.m^{-2}.s^{-1}), the liquid culture was blended and spread onto the surface of Potato Dextrose Agar (PDA) 12 g.L^{-1} medium. *Bc*630 was incubated for 3 weeks at 20 °C until mycelium growth and spores production. For plant infection, spores were collected in Potato Dextrose Broth (PDB) 12 g.L^{-1} medium by scraping surface of the mycelium with a sterile loop. Suspension concentration was adjusted to 10^5 conidia.mL^{-1} after counting using Malassez cell. Spores suspension was incubated at 20 °C and 140 rpm for 3 h to initiate germination.

2.2. Molecules

Ananatoside A (An.A) and ananatoside B (An.B), biosurfactants secreted by *P. ananatis* BRT175 (Gauthier et al. 2019) were used in this study, primarily in antifungal assays. Molecules were synthetized using the procedure detailed by Cloutier et al. (2021). Both molecules were diluted at 20 mM in methanol and conserved at −20 °C.

2.3. Plant growth conditions and bacterial inoculation

Tomato (*Solanum lycopersicum* cv. Ailsa Craig) seeds were sown in pot (15 cm x 15 cm x 20 cm) containing 150 g of non-sterile soil (Gramoflor). Seedlings were placed in growth chamber (24 °C /16 h light and 20 °C / 8 h dark). The light intensity was set at 200 μmol.m^{-2}.s^{-1} and the relative humidity was maintained at 60 %.

Three-and-a-half-week-old tomato plants were soil-drenched with *P. ananatis* BRT175 suspension at 10^8 CFU.g^{-1} of soil, or with sterile 10 mM $MgSO_4$ for the mock-treated plants. These plants were used for growth promotion experiments and systemic protection assays. For local protection assays, leaves were sprayed at 4.5 weeks with *P. ananatis* BRT175 suspension at 10^8 CFU.mL^{-1}, or with sterile 10 mM $MgSO_4$ for mock-treated plants.

For longer experiments, plants were repotted (into 20 cm diameter pots) and transferred to a greenhouse (24 °C/16 h light and 20 °C/8 h dark, relative humidity maintained at 55 %) two weeks after the first inoculation. Plants were inoculated a second time at the same concentration (10^8 CFU.g^{-1} of soil, or with sterile 10 mM $MgSO_4$), 2 days after repotting.

2.4. Phosphate solubilization

The bacterial suspension of *P. ananatis* BRT175 was prepared as previously mentioned and diluted to a final bacterial concentration of 10^6 CFU.mL^{-1}. Four drops of 5 μL bacterial suspension were inoculated on Petri dish containing phosphate-rich National Botanical Research Institute's Phosphate (NBRIP) medium (glucose 10 g.L^{-1}, $Ca_3(PO_4)_2$ 5 g.L^{-1}, $MgCl_2{\cdot}6H_2O$ 5 g.L^{-1}, $MgSO_4{\cdot}7H_2O$ 250 mg.L^{-1}, KCl 200 mg.L^{-1}, $(NH_4)_2SO_4$ 100 mg.L^{-1}). Petri dishes were then sealed with parafilm and incubated at 28 °C for 24 h. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Phosphate solubilization index (PSI) was calculated using the following formula:

$$\mathrm{PSI} = \frac{\text{Diameter of transparent halo}}{\text{Diameter of bacterial spot}}$$

2.5. Nitrogen-fixing ability

P. ananatis BRT175 was spread on nitrogen-deprived Jensen's medium (sucrose 20 g.L^{-1}, $CaCO_3$ 2 g.L^{-1}, $MgSO_4$ 500 mg.L^{-1}, K_2HPO_4 1 g.L^{-1}, NaCl 500 mg.L^{-1}, $FeSO_4$ 100 mg.L^{-1}, Na_2MoO_4 5 mg.L^{-1}, Agar 15 g.L^{-1}, pH 6.8). To allow gas exchange, the Petri dishes were not sealed with parafilm. Plates were then incubated at 28 °C for 24 h. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using

a similar procedure. Growth of colony on this nitrogen-deprived medium indicates a capacity to fix atmospheric nitrogen.

2.6. Siderophore production

The bacterial suspension of *P. ananatis* BRT175 was prepared as previously mentioned and diluted to a final bacterial concentration of 10^6 CFU.mL^{-1}. Four drops of 5 µL bacterial suspension were inoculated on Petri dish containing CAS (Chrome Azurol S) medium. Media were prepared as follows: the staining solution was firstly prepared by mixing 50 mL of CAS (1.21 g.L^{-1}), 9 mL of $FeCl_3$ (16.2 mg.L^{-1} with 13 N HCl, 830 µL.L^{-1}) and HDTMA (1.82 g.L^{-1}). Staining solution was then diluted at 5 % in LB medium with 15 % agar. Petri dishes were sealed with parafilm and incubated at 28 °C for 24 h. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Siderophore index (SI) was calculated using the following formula:

$$SI = \frac{\text{Diameter of orange halo}}{\text{Diameter of bacterial spot}}$$

2.7. Indole acetic acid production

An overnight preculture of *P. ananatis* BRT175 strain in nutrient broth (glucose 1 g.L^{-1}, peptone 15 g.L^{-1}, NaCl 6 g.L^{-1}, yeast extract 3 g.L^{-1}) was used to inoculate a tryptophan-rich nutrient broth (identical medium supplemented with tryptophan 1.5 g.L^{-1}). At 24, 48, 72 and 96 hpi, 2 mL of bacteria culture were collected, transferred to a 2 mL Eppendorf tube, and centrifuged at 3500 *g* at 8° C for 15 min. One milliliter of supernatant was transferred to a new 2 mL Eppendorf tube. Salkowski reagent ($FeCl_3$ 12 g.L^{-1} in 7.9 M H_2SO_4) was added to the supernatant (v/v). Tubes were wrapped in aluminum foil and incubated in the dark for 30 min at 30 °C with 180 rpm agitation. To quantify IAA synthesis, a standard curve was generated using uninoculated medium supplemented with known concentrations of 3-indole acetic acid, and absorbance was measured at 536 nm using a spectrophotometer.

2.8. Tomato plant growth assessment

The length of root-inoculated and control tomato plants was measured from the cotyledons to the tip of the last expanded leaf 10 days after root treatment. The fresh weight of the aerial parts of plants was recorded directly after length measurements. Samples were dried at 65 °C for 4 days to determine the dry weight. Longer experiments were also performed in greenhouse. Lengths were measured 14 days after repotting.

2.9. Measurement of chlorophyll content

Chlorophyll content in tomato leaves was evaluated 10 days after root inoculation. Chlorophyll measurements were performed on the third and fourth leaves of both root-inoculated and control plants using a SPAD-502Plus chlorophyll meter (Konica Minolta).

2.10. Antifungal tests

The bacterial suspension of *P. ananatis* BRT175 was prepared as previously mentioned and diluted to a final bacterial concentration of 10^8 CFU.mL^{-1}. Four drops of 5 µL of bacterial suspension were inoculated on Petri dish containing PDA medium (24 g.L^{-1}). Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Plates were incubated at 20 °C for 24 h. A 15 µL drop of Bc630 conidia suspension (10^5 conidia.mL^{-1}) was then placed in the center of the plate. Pictures were taken after 6 days of incubation at 20 °C. For assays involving ananatosides, molecules were incorporated directly in PDA medium at a final concentration of 100 µM. Methanol (0.5 %) was used as negative control. Assays were realized in sterile 6-well plates. Pictures were taken after 4 days of incubation at 20 °C. The relative inhibition (RI) of *Bc*630 growth was calculated using the following formula:

$$RI = \frac{(\text{radial growth of control} - \text{radial growth of bacteria test})}{\text{radial growth of control}} \text{x } 100$$

To assess the activity of ananatosides against fungal spores, assays were conducted in sterile 96-well plates. The conidial suspension was prepared at 5 x 10^4 conidia.mL^{-1}, and 100 µL of this suspension was dispensed into each well. Molecules were then added directly to the suspension at a final concentration of 100 µM. After 16 h of incubation at 20 °C, images of each well were captured using an FL-EVOS inverted microscope (Thermo Fisher Scientific).

2.11. Protection assays

For systemic protection assays, the third and fourth leaves of root-inoculated and control tomato plants were collected 10 days after root treatment and placed in Petri plates containing 0.75 % agar. For local protection assays, leaves were detached 2 days after inoculation. A 10 µL drop of *Bc*630 spores suspension (10^5 conidia.mL^{-1}) was applied to the central vein of the adaxial surface of each leaf. Leaves were then incubated in a growth chamber at 20 °C with 100 % relative humidity. The diameter of fungal lesions was measured 96 h after infection.

2.12. Bioinformatic analysis

GenBank annotated genes of *P. ananatis* BRT175 (GCA_000475035.1) were used as input for BlastKOALA (Kanehisa et al. 2016) to identify genes associated with beneficial traits of PGPR.

2.13. Statistical analysis

Statistical analyses were conducted using GraphPad Prism v9.0. Unless otherwise specified, statistical differences of means were tested using Student tests after confirming normal distribution with Shapiro-Wilk tests. Differences were considered statistically significant at $p \leq 0.05$. Each experiment was independently repeated twice, corresponding to two biological replicates. For each biological replicate, the number of technical replicates (n) is indicated in figure captions.

3. Results

3.1. P. ananatis BRT175 possesses genes associated with PGPR traits

Analysis of *P. ananatis* BRT175 genome performed on BlastKOALA revealed genes involved in the different PGPR functions (Table 1 and Fig. 1). Phosphate solubilization is for instance correlated with the presence of genes encoding for phosphatase (*phoA*) or organic acid synthesis (*pqqABCDE* cluster and gene encoding a glucose dehydrogenase). We also identified the complete biosynthetic cluster of the siderophore aerobactin (*iucABCD*) and genes responsible for indole-3-pyruvate synthesis (aromatic amino acid aminotransferase), indole-3-acetaldehyde synthesis (*ipdC*) and indole-3-acetic acid (*aldh*).

3.2. P. ananatis BRT175 exhibits plant growth-promoting features

We investigated the ability of *P. ananatis* BRT175 to solubilize inorganic phosphate (Pi), to fix atmospheric nitrogen, and to produce siderophore and indole-3-acetic acid (IAA), four key features typically associated with PGPR. The capacity of *P. ananatis* BRT175 to solubilize Pi, under the form of tricalcium phosphate in the NBRIP medium, was evaluated by measuring a halo of solubilization around the spot of bacteria (Fig. 2A). No halos were detected in the negative control

Table 1
Annotated genes of *P. ananatis* BRT175 associated with PGPR activity.

PGPR features	Gene name	Genbank ID	Encoded protein
Phosphate solubilization	*NA*	ERM15599.1	Glucose dehydrogenase
	pqqA	*NA**	Pyrroloquinoline quinone precursor peptide
	pqqB	ERM13961.1	Pyrroloquinoline quinone biosynthesis protein B
	pqqC	ERM13962.1	Pyrroloquinoline-quinone synthase
	pqqD	ERM14089.1	Pyrroloquinoline quinone biosynthesis protein D
	pqqE	ERM13963.1	PqqA peptide cyclase
	phoA	ERM14005.1	Alkaline phosphatase
Siderophore synthesis	*iucA*	ERM15632.1	N2-citryl-N6-acetyl-N6-hydroxylysine synthase
	iucB	ERM15633.1	Acetyl CoA:N6-hydroxylysine acetyl transferase
	iucC	ERM15634.1	Aerobactin synthase
	iucD	ERM15635.1	Lysine N6-hydroxylase
IAA synthesis	*NA*	ERM15558.1	Aromatic amino acid aminotransferase
	NA	ERM11656.1	Aromatic amino acid aminotransferase
	ipdC	ERM12274.1	Indolepyruvate decarboxylase
	aldH	ERM14037.1	Aldehyde dehydrogenase

*: *pqqA* was not identified in the annotated genome of *P. ananatis* BRT175. BlastN with the sequence of *pqqA* gene from *P. ananatis* PA13 (NC_017554.1:2443649–2443720) allows the identification of this gene at position ASJH01000012.1 (83,725..83,796).

inoculated with $MgSO_4$. In comparison to *E. coli*, a significant higher PSI was measured with *P. ananatis* BRT175 (Fig. 2B), indicating a capacity of phosphate solubilization by this strain. Siderophore production was tested by inoculating CAS medium with the two bacteria. Clear orange halos appeared around bacterial spots of *P. ananatis* BRT175 but also around *E. coli* (Fig. 2C). Such halos were absent in the negative control inoculated with $MgSO_4$. However, a statistical analysis of the siderophore index reveals that *P. ananatis* BRT175 presents a significant higher effect, in comparison to *E. coli* (Fig. 2D). Atmospheric nitrogen fixation was then studied by spreading *P. ananatis* BRT175 on the nitrogen-deprived Jensen medium (Fig. 2E). After 24 h of incubation, no bacterial colonies development was observed, neither for *P. ananatis* BRT175 nor for *E. coli*. No bacterial growth was also observed on medium inoculated with $MgSO_4$. We therefore assumed that this strain is not able to fix N_2. Lastly, IAA production by the bacteria was evaluated over a time course of 72 h (Fig. 2F) through the reaction of this molecule with the Salkowski reagent. Over the analysis, an increase of IAA concentration was measured in the culture supernatant, reaching a final value of approximately 110 $\mu g.mL^{-1}$, indicating the capacity for *P. ananatis* BRT175 to secrete this compound.

3.3. *P. ananatis BRT175 promotes plant growth*

Since *P. ananatis* BRT175 displayed *in vitro* plant-growth promoting characteristics, we then investigated whether *P. ananatis* BRT175 is able to enhance tomato development when applied at the root level. *In planta* assays were performed through measurements of aerial parts length, fresh and dry weights following a soil-drench application of bacteria. Plants that were root-treated with *P. ananatis* BRT175 were significantly taller than mock-treated tomatoes (Fig. 3A). An increase of fresh weight was also observed for bacteria-treated tomatoes (Fig. 3B). However, no significant differences were observed for dry weight between control and bacterized conditions (Fig. 3C). The same experiment was conducted in greenhouse with two successive *P. ananatis* BRT175 soil inoculation. After 14 days in greenhouse, root-bacterized tomatoes were significantly taller (more than 10 cm in average) in comparison with mock-treated plants (Figs. 3D and 3E). Measurements using SPAD also reveal that root-treatments with *P. ananatis* BRT175 increase the chlorophyll content in both the third and fourth leaf of tomato (Supplemental Fig. 1).

3.4. *P. ananatis BRT175 and its ananatosides exhibit antifungal effects*

The direct antifungal effects of live *P. ananatis* BRT175 as well as its two amphiphilic produced metabolites, ananatoside A (An.A) and B (An. B) were evaluated against *B. cinerea*, the causing agent of grey mold (Figs. 4A and 4B). A significant reduction of mycelial growth was measured (around 50 %) when *B. cinerea* was co-cultured with *P. ananatis* BRT175, while $MgSO_4$ and *E. coli* had no effect on fungal development, indicating a clear antifungal effect of *P. ananatis* BRT175.

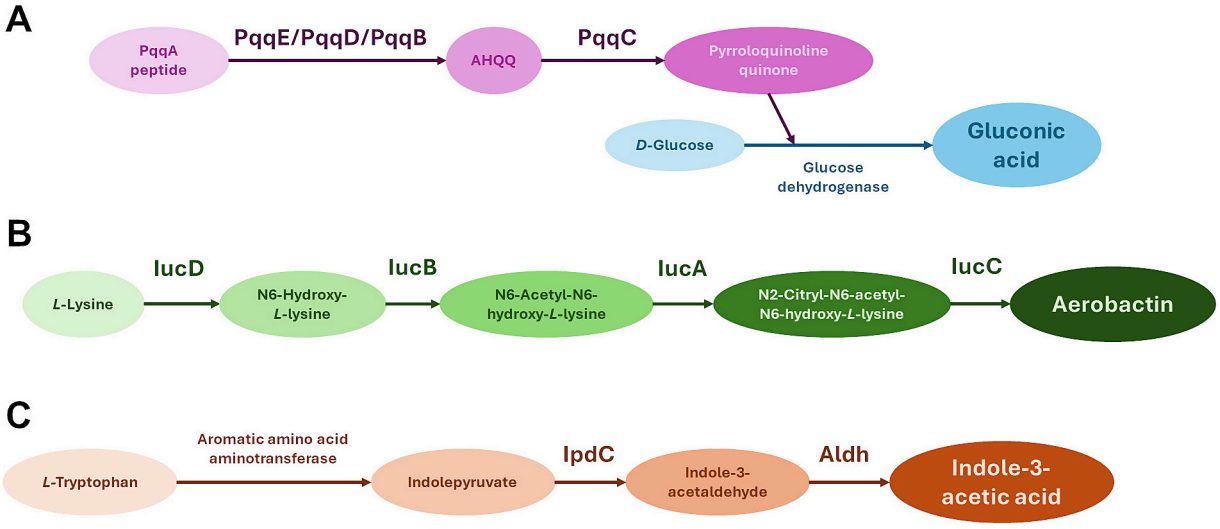


Fig. 1. Biosynthetic pathways of compounds involved in PGPR traits of *P. ananatis* BRT175. (A) Biosynthesis of gluconic acid, participating in soil acidification and inorganic phosphate solubilization. Pyrroloquinoline quinone is the co-factor of the glucose dehydrogenase formed by enzymes encoded by *pqqBCDE* genes. It is synthetized from 3a-(2-amino-2-carboxy-ethyl)-4, 5-dioxy-4, 6, 7, 9-hexahydroartemisinin-7, 9-dicarboxylic acid (AHQQ) itself formed from the peptide encoded by *pqqA*. (B) Biosynthesis of aerobactin. The synthesis of this siderophore starts from *L*-lysine and involves enzymes encoded by genes of the *iucDBAC* cluster. (C) Biosynthesis of indole-3-acetic acid. The synthesis of this compound, acting as a hormone for plants, starts from *L*-Tryptophan and involves an aromatic amino acid aminotransferase alongside enzymes encoded by *ipdC* and *aldh*.

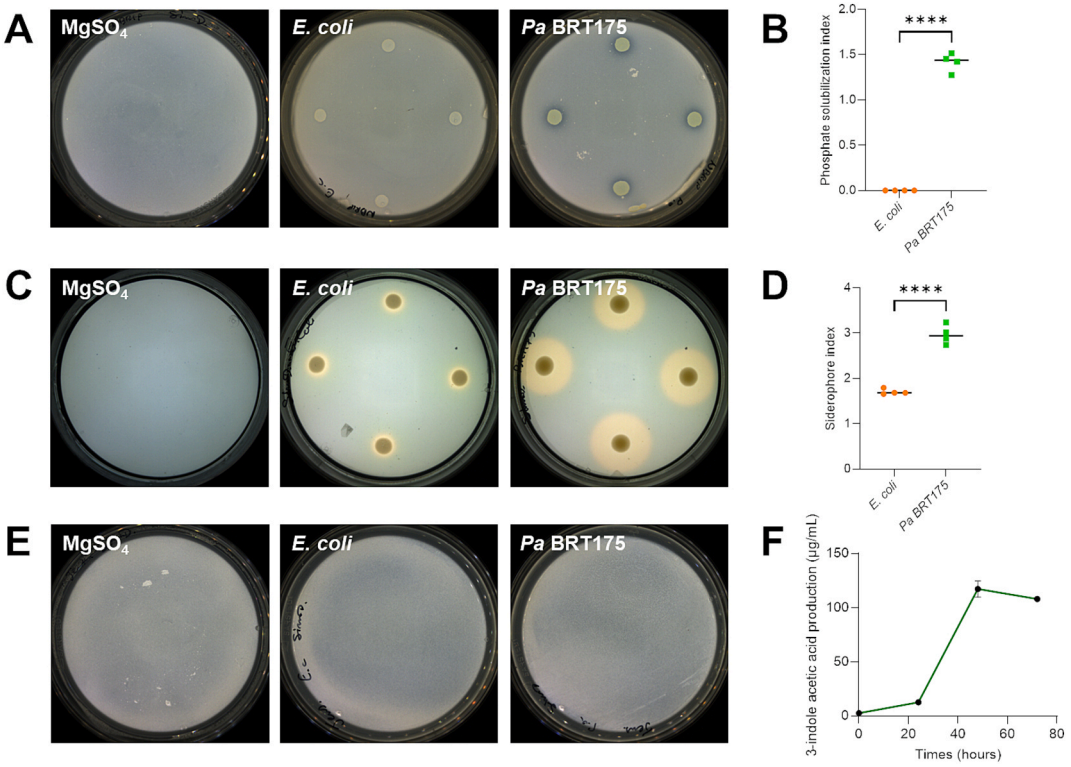


Fig. 2. *In vitro* characterization of *P. ananatis* BRT175 PGPR features. (A) Solubilization of inorganic phosphate was assayed on NBRIP medium. *P. ananatis* (*Pa*) BRT175 was inoculated on media with four 5 µL drops at 10^6 CFU.ml^{-1}. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Pictures were taken after 24 h of incubation at 28 °C. Phosphate solubilization index (B) was calculated with the following ratio: Halo diameter / Bacterial spot diameter. Data from a representative biological replicate are plotted individually (n = 4, black bar: mean). The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. (C) Siderophore synthesis was assayed on CAS medium. Bacteria were inoculated on media with four 5 µL drops at 10^6 CFU.ml^{-1}. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Pictures were taken after 24 h of incubation at 28 °C. Siderophore index (D) was calculated with the following ratio: Halo diameter / Bacterial spot diameter. Data from a representative biological replicate are plotted individually (n = 4, black bar: mean). The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. (E) Fixation of atmospheric nitrogen was tested with the Jensen medium, upon which bacteria were spread. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Pictures were taken after 24 h of incubation at 28 °C. (F) Kinetic of indole-3-acetic acid production by *P. ananatis* BRT175. Bacteria were inoculated in a tryptophane-supplemented growth medium. Optical densities of supernatant mixed with Salkowski's reagent were measured at $\lambda = 536$ nm. Concentration values were obtained using a standard curve at 0, 24, 48 and 72 hpi (n = 3). Data from a representative biological replicate are plotted individually (n = 3). Experiments were independently repeated twice with similar results.

Since this strain is known to produce An.A and An.B, the putative antifungal effect of these two amphiphilic molecules was analyzed. *B. cinerea* was cultivated on PDA medium containing either An.A or An.B at 100 µM final concentration (Figs. 4C and 4D). In comparison to control medium (containing methanol), a significant reduction of mycelial development was observed for both compounds. Indeed, An.A reduces growth by approximately 30 % while An.B exhibits a significantly stronger antifungal effect, reducing the development of *B. cinerea* by 80 %. Additional experiments conducted on spores (Supplemental Fig. 2) clearly showed that An.B alters the hyphal development of *B. cinerea*. On the contrary, the effect of An. A on hyphae were not detectable. Altogether these data indicate that *P. ananatis* BRT175 presents direct antifungal properties against *B. cinerea*, as well as the ananatosides produced by this strain.

3.5. *P. ananatis BRT175 enhances local and systemic resistance against B. cinerea*

Regarding the previously observed antifungal properties of *P. ananatis* BRT175, we investigated the local protection of tomato leaves by spraying directly the bacteria on leaves, 2 days prior infection with *B. cinerea*. A significant reduction of necrotic area was observed on tomato leaves treated with *P. ananatis* BRT175 compared to control conditions (Figs. 5A and 5B). Indeed, lesion size was reduced from an average of approximately 0.8 cm^2 to less than 0.2 cm^2. It is well-known that beneficial bacteria can also protect plants from diseases by inducing systemic responses (ISR). To decipher the capacity of *P. ananatis* BRT175 to induce systemic resistance in tomato against *B. cinerea*, bacteria were inoculated in soil, in the rhizospheric area. Ten-days after inoculation, the leaves of tomato were infected with *B. cinerea* conidia. In comparison with mock-treated plants, leaves of root-treated tomato were significantly less susceptible to *B. cinerea*. Necrotic areas triggered by this fungus (Fig. 6A) were reduced from 6 cm^2 to 3.5 cm^2 (Fig. 6B) in inoculated plants. Altogether, our results indicate that *P. ananatis* BRT175 is able to protect tomato plants against the fungus at the local and systemic levels.

4. Discussion

Bacteria from the *Pantoea* genus have been frequently described as endophytes and efficient PGPR among diverse plant species like wheat (Ansari et al. 2024; Chen et al. 2017), sugarcane (Singh et al. 2021; Quecine et al. 2012), maize (Mishra et al. 2011) or chickpea (Mishra et al. 2011). Alongside these effects on plant growth, numerous studies have characterized the biocontrol potential of this genus. In particular, *Pantoea* bacteria are well-known for their capacities to protect plants

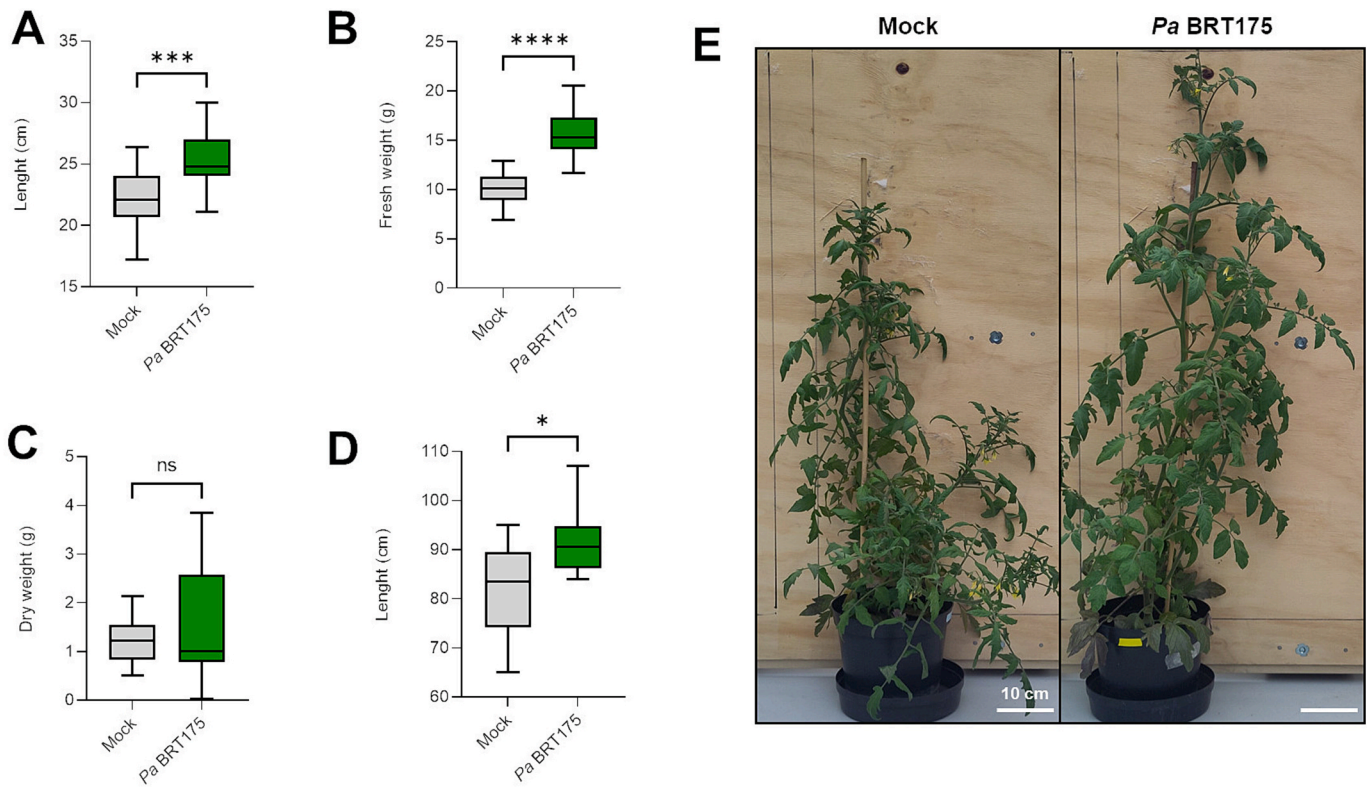


Fig. 3. Impact of *P. ananatis* BRT175 on the morphology of tomato plants. Tomato roots were inoculated with *P. ananatis* (*Pa*) BRT175 (10^8 CFU.g^{-1} of soil) or treated with 10 mM $MgSO_4$ (mock control) after 3.5 weeks of growth. (A) Length, (B) fresh weight and (C) dry weight were measured 10 days after inoculation. Length was measured from the cotyledons to the tip of the last unfolded leaf. Regarding dry weights, aerial parts were dried at 65 °C for 4 days, then weighed. The box plots represent data from a representative biological replicate with minimum n = 13 for each condition. Statistical differences were assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. The experiment was independently repeated twice with similar results. (D) The same experiment was conducted in greenhouse, where length of plants was measured 14 days after repotting. The box plots represent data from a representative biological replicate with n = 10 for each condition. The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). The asterisk indicates statistically significant differences. (E) Pictures of tomato grown in greenhouse taken 14 days after repotting.

against pathogens like *Erwinia amylovora* (Pusey et al. 2011), *Monilinia laxa* (Lahlali et al. 2020, Bonaterra et al. 2003), *Pseudomonas syringae* pv. *tomato* (Morella et al. 2019) or *Burkholderia glumae* (Kouzai and Akimoto-Tomiyama, 2022). *P. ananatis* strains, while less described than other *Pantoea* species like *P. agglomerans*, have also been identified as promising promoters of growth in rice (Lu et al. 2021), pepper (Kang et al. 2007) and quinoa (Valbuena-Rodríguez et al. 2024) for instance. This species also illustrates itself as a prospective biocontrol agent against *B. cinerea* (Gasser et al. 2012), *E. amylovora* (Lee et al. 2024) or *Xanthomonas axonopodis* pv. *vesicatoria* (Kang et al. 2007).

Our results showed the capacity of *P. ananatis* BRT175 to solubilize Pi, a feature shared by other strains of the species (Valbuena-Rodríguez et al. 2024; Liu et al. 2021). Mechanisms by which *P. ananatis* BRT175 retrieve phosphate from inorganic sources were explored through bioinformatic analysis. On a global standpoint, bacteria generally solubilize Pi through soil acidification, enabled by the synthesis of organic acids (Billah et al. 2019). Secretion of phosphatase is another mechanism by which bacteria can solubilize Pi (Billah et al. 2019). Bioinformatic analysis revealed that *P. ananatis* BRT175 does possess the *pqqABCDE* cluster and a gene encoding a glucose dehydrogenase (GDH). Pqq enzymes enable the synthesis of tricarboxypyrroloquinoline quinone, a cofactor of GDH (Lisdat, 2020). The reaction catalyzed by GDH leads to the synthesis of gluconic acid, a compound which is highly involved in Pi solubilization by soil acidification (Billah et al. 2019). In addition, BlastKOALA reveals the existence of an alkaline phosphatase encoding gene, *phoA*, in the genome of *P. ananatis* BRT175. The related enzyme is known to play an important role in hydrolysis of inorganic phosphate (Pan and Cai, 2023). In this work, we also highlighted the production of siderophores by *P. ananatis* BRT175. Synthesis of such metabolites has already been witnessed in *P. ananatis* strains (Choi et al. 2022; Lu et al. 2021). The complete biosynthetic cluster of aerobactin has been identified by BlastKOALA in the genome of *P. ananatis* BRT175. Interestingly, we also observed the production of siderophores by *E. coli* used as a control. In fact, *E. coli* has already been described as a siderophore producer (Cavas and Kirkiz, 2022). However, the potential of iron capture deployed by *P. ananatis* BRT175, was significantly greater in comparison to *E. coli*. We also found that *P. ananatis* does not have the capacity to fix N_2. To fix N_2, diazotrophic bacteria mostly rely on nitrogenase, generally encoded by *nif* (nitrogen fixation) gene (Raymond et al. 2004). Interestingly, *nif* genes were not found in the annotated genome of *P. ananatis* BRT175. In addition, nitrogenase using vanadium, encoded by *vnf* genes, can be found in diazotrophic bacteria, but such genes were not found in *P. ananatis* BRT175, that could also explain the inability of the strain to fix N_2. Lastly, we measured a constant production of indole by *P. ananatis* BRT175. Multiple types of indoles can be detected by the method we used here (Guardado-Fierros et al. 2024). By exploring the annotated genes of *P. ananatis* BRT175, it appears that this strain possesses the *ipdC* gene, encoding an indolepyruvate decarboxylase, and an *aldh* gene, coding for an aldehyde dehydrogenase. Both enzymes are successively involved in the latter steps of IAA biosynthesis from tryptophan. Surprisingly, BlastKOALA did not retrieve tryptophan aminotransferase in the genome of *P. ananatis* BRT175, the first enzyme of the pathway which catalyzes the synthesis of indolepyruvate from tryptophan. However, two aromatic amino acid

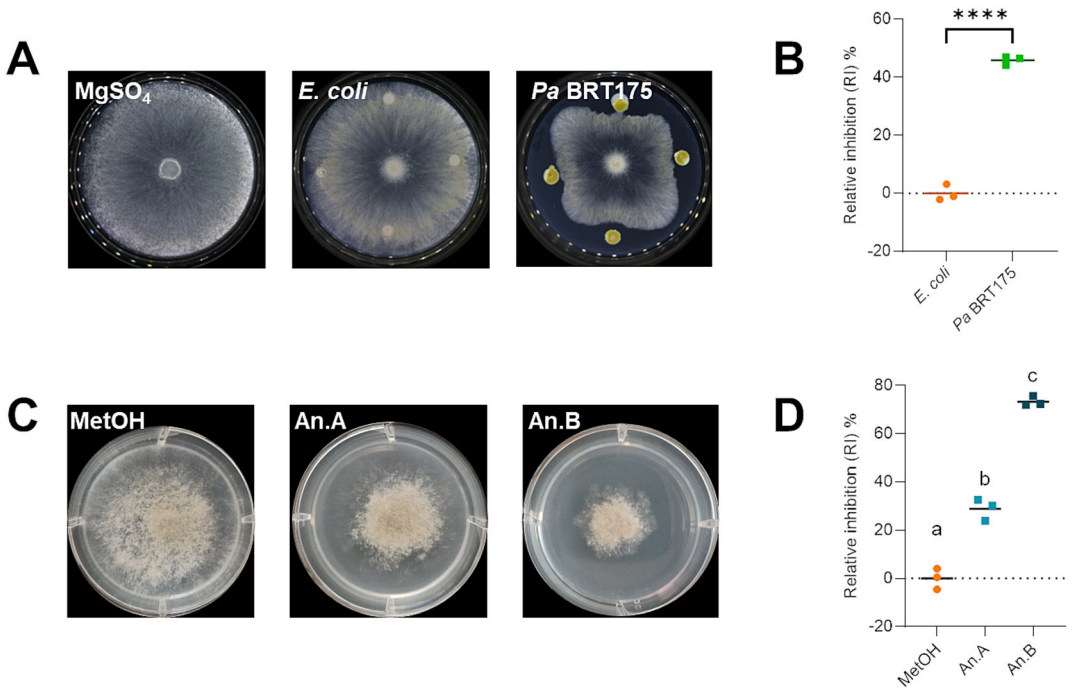


Fig. 4. Direct antifungal activity of *P. ananatis* BRT175 and ananatosides on the growth of *B. cinerea*. (A) The antifungal effect of *P. ananatis* (*Pa*) BRT175 was assessed against *B. cinerea*. *P. ananatis* (*Pa*) BRT175 was co-cultivated with *B. cinerea* (10^5 conidia.mL^{-1}) on PDA medium. Negative controls were inoculated with sterile 10 mM $MgSO_4$ or with *E. coli* using a similar procedure. Pictures were taken after 6 days of incubation. (B) Plot representing the percentage of relative inhibition of *B. cinerea* mycelial growth by *Pa* BRT175. Data from a representative biological replicate are plotted individually (n = 3, black bar: mean). The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. (C) The antifungal effect of ananatosides was assessed against *B. cinerea.* PDA medium containing ananatosides (form A or B, final concentration of 100 μM) was inoculated with *B. cinerea* (10^5 conidia.mL^{-1}). Methanol (0.5 %) was used as a negative control. Pictures were taken after 4 days of incubation. (D) Plot representing the relative inhibition of *B. cinerea* mycelial growth by ananatosides. Data from a representative biological replicate are plotted individually (n = 3, black bar: mean). Statistical differences were assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Different letters indicate significant differences. Experiments were independently repeated twice with similar results.

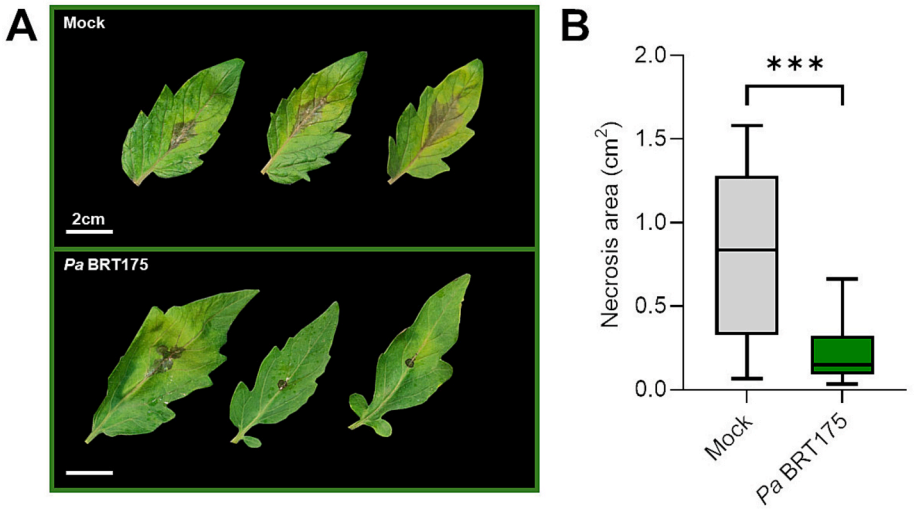


Fig. 5. Local bioprotection test against *B. cinerea* on detached leaves of leaf-treated tomato plants. Tomato leaves were sprayed with *P. ananatis* (*Pa*) BRT175 (10^8 CFU.mL^{-1}) or 10 mM $MgSO_4$ (mock control), after 4.5 weeks of growth. After 2 days, leaflets from third and fourth leaves were detached, inoculated with 10 μL of a suspension of *B. cinerea* conidia (10^5 conidia.mL^{-1}) and incubated at 20 °C. Pictures of leaves were taken 96 h after infection (A), then, necrotic areas (B) were measured using ImageJ. The box plots represent data from a representative biological replicate with minimum n = 18 for each condition. The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. The experiment was independently repeated twice with similar results.

aminotransferases are present in the annotated genome of the bacteria. Such enzymes, with a broad spectrum of substrate, could possibly carry the first step of the biosynthetic pathway (Patten et al. 2013), thus explaining IAA synthesis in *P. ananatis* BRT175.

Interestingly, *P. ananatis* BRT175 also exhibits *in planta* PGPR effects. Inoculation of *P. ananatis* BRT175 in tomato rhizosphere significantly

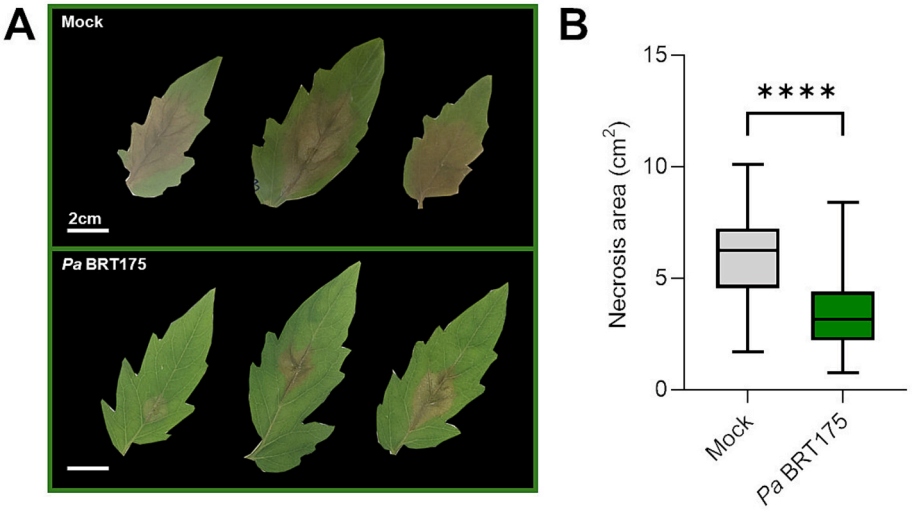


Fig. 6. Systemic bioprotection test against *B. cinerea* on detached leaves of root-bacterized tomato plants. Tomato roots were inoculated with *P. ananatis* (*Pa*) BRT175 (10^8 CFU.g^{-1} of soil) or treated with 10 mM $MgSO_4$ (mock control) after 3.5 weeks of growth. After 10 days, leaflets from third and fourth leaves were detached, inoculated with 10 μL of a suspension of *B. cinerea* conidia (10^5 conidia.mL^{-1}) and incubated at 20 °C. Pictures of leaves were taken 96 h after infection (A), then, necrotic areas (B) were measured using ImageJ. The box plots represent data from a representative biological replicate with minimum n = 38 for each condition. The statistical difference was assessed using a Student test ($p \leq 0.05$) after confirming normal distribution by a Shapiro-Wilk test ($p > 0.05$). Asterisks indicate statistically significant differences. The experiment was independently repeated twice with similar results.

promotes plant development, both in growth chamber and in greenhouse. Among the different morphological measures, a significant increase of length and fresh weight was observed. Since dry weights are identical in both treated and mock plants, the increase of length appears linked with an increase of the water content in tomato cells. As a phytohormone, IAA is known to promote cell elongation in plants, mostly by activation of cell-wall modifying enzymes through apoplast acidification (Majda and Robert, 2018; Velasquez et al. 2016). Previous studies have already revealed that IAA-producing bacteria can increase shoot fresh weight and length when introduced in the rhizosphere of tomato (Aydi Ben Abdallah et al. 2016; Gravel et al. 2007). We thus suggest that IAA synthesis by *P. ananatis* BRT175 may play a prevalent role in the growth promotion of tomato. Additionally, we also witnessed an increase in the chlorophyll content of tomato leaves following root application of *P. ananatis* BRT175. This increase could be a consequence of an improved nutrients uptake (Su et al. 2024), but in return, it could also enhance tomato photosynthesis and therefore plant growth.

In this study, the biocontrol capacity of *P. ananatis* BRT175 was characterized. Direct confrontation of *P. ananatis* BRT175 with *B. cinerea* significantly reduces mycelial growth of the fungus. This is consistent with other studies showing that several *P. ananatis* strains have the ability to inhibit growth of this fungus (Gasser et al. 2012) but also to other fungi (Valbuena-Rodríguez et al. 2024). *P. ananatis* BRT175 secrete glycolipid analogues to rhamnolipids called ananatosides (Cloutier et al. 2021; Gauthier et al. 2019). As rhamnolipids have well known antifungal activities, especially against *B. cinerea* (Crouzet et al. 2020), we investigated whether ananatosides display an antifungal effect and could therefore be putatively involved in the antagonism effect of this strain. Both An.A and An.B are able to limit the mycelial growth of the fungus when directly added to the medium. Amphiphilic compounds including glycolipids but also lipopeptides are known to strongly impede fungal development (Crouzet et al. 2020; Mnif and Ghribi, 2016; Cawoy et al. 2015). Interestingly, we observed that An.B effect was significantly greater compared to An.A. We also observed a difference between An.A and An.B regarding their effects on hyphal development of *B. cinerea*. In fact, the addition of An.B in the culture medium drastically altered hyphae growth. On the contrary, An.A did not visually inhibit the germination of *B. cinerea* spores. The A form presents a 15-membered macrodilactone ring (Cloutier et al. 2021; Gauthier et al. 2019), while the B form is structurally close to rhamnolipids. These structural changes may have repercussions on the polarity of these two ananatosides. Biological activities of amphiphilic compounds are possibly driven by their capacity to interact with plasma membrane (Botcazon et al. 2022; Crouzet et al. 2020; Henry et al. 2011), and thus their capacity to interact with lipidic bilayers. Robineau et al. (2020) have previously demonstrated that even slight alterations of structures in glycolipids significantly modify their antifungal effect. The macrodilactone ring present in An.A thus may limit the antifungal potential of this molecule.

The capacity of *P. ananatis* BRT175 to protect tomato against *B. cinerea* at both local and systemic scales was also characterized. When applied as root drench, *P. ananatis* BRT175 was able to protect tomato leaves against the fungus, thus illustrating its capacity to trigger a systemic defense response. The ability to trigger ISR is a very efficient mechanism by which soil bacteria protect plants. Bacteria from the *Pseudomonas* or *Bacillus* genus are for instance characterized as excellent inducers of systemic resistance (Nimbeshaho et al. 2024; Nguyen et al. 2022; Takishita et al. 2018). Such ability is valuable for biocontrol strategies as it enhances plant defense response at a low fitness cost through priming (Martinez-Medina et al. 2016; Conrath et al. 2015). This capacity to trigger systemic resistance has been observed for several *Pantoea* spp. in diverse plant species like rice (Spence et al. 2014) or grapevine (Magnin-Robert et al. 2013), but also in tomato (Zheng et al. 2025). However, this capacity has been poorly described for the *P. ananatis* species as it has only been demonstrated in pepper (Kang et al. 2007). Upon these previous observations, our data thus confirm the potential of the *P. ananatis* species as a systemic resistance inducer in crops. Interestingly, similar results were observed with local leaf treatment. Necrotic areas were significantly smaller in *P. ananatis* BRT175-treated tomato leaves. As *P. ananatis* BRT175 was directly confronted to *B. cinerea* in this experiment, the antifungal effect displayed by this strain and its ananatosides could play a significant role in the observed protection. Siderophore production could also participate in this direct protection effect, as it plays a role in nutrient competition (Kramer et al. 2020). Lastly, the local defense response induction in tomato leaves could also be involved in the protection effect of *P. ananatis* BRT175. This hypothesis is supported by the fact that ananatosides are known to be sensed by tomato leaves and to trigger a plant immune response (Cloutier et al. 2021).

In conclusion, our results demonstrate that *P. ananatis* BRT175 is a prospective multifaceted tool for plant growth stimulation and plant

protection. We showed that *P. ananatis* BRT175 enhances the growth of aerial parts of tomato plants. At the same time, we observed that this strain exhibits several growth-promoting traits including phosphate solubilization, siderophore synthesis and IAA production. This combination of beneficial features could be involved in the effect of *P. ananatis* BRT175 on plant growth. Treated plants also tend to be less susceptible to pathogen aggression, both at the local and systemic levels. The different protective mechanisms displayed by *P. ananatis* BRT175, including the production of antifungal amphiphilic molecules and the induction of the plant defense response, could participate in tomato protection. Altogether, these results make *P. ananatis* BRT175 a bacterium that can potentially be integrated into biocontrol strategies. Regarding their implication in the antifungal effect of this strain, ananatosides also appear to be valuable compounds for crop protection.

CRediT authorship contribution statement

Simon Duchateau: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Célia Borrego:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Sonia Verdelet:** Investigation, Formal analysis. **Stéphan Dorey:** Writing – review & editing, Validation, Conceptualization. **Aziz Aziz:** Writing – review & editing, Validation, Conceptualization. **Sandrine Dhondt-Cordelier:** Writing – review & editing, Validation, Conceptualization. **Charles Gauthier:** Writing – review & editing, Validation, Resources. **Éric Déziel:** Writing – review & editing, Validation, Resources. **Sylvain Cordelier:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Jérôme Crouzet:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by grants from the MESRI (Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation), from the Federative Research Structure SFR Condorcet and from ANR-21-ECOM-0006 (SHIELD project).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2025.105949.

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